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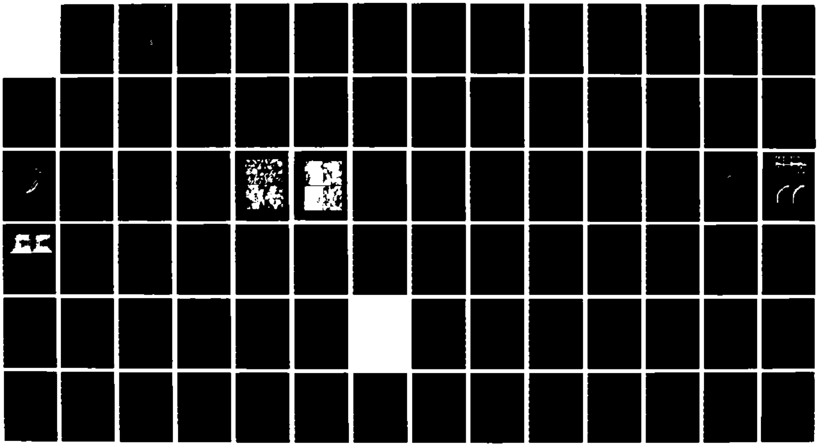

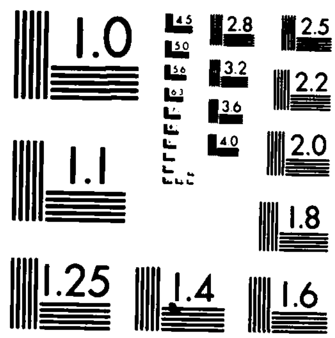

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BY

WAYNE ROBERT PATTERSON

AN ABSTRACT OF A DISSERTATION

SUBMITTED TO
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in partial fulfillment of the requirements
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TO DEBBIE AND MEGHAN

ACKNOWLEDGEMENTS

Any verbal or written expression of gratitude to Dr. Thomas Graham Bell would be inadequate. As chairman of my guidance committee and my research advisor, he was continually there with encouragement, advice, and new ideas. He showed me all the aspects of what a PhD means in terms of thinking through the problem, performing the research, and achieving my research goals. For his invaluable advice and insight I am eternally grateful.

Special thanks are also deserving to the other members of my committee. Dr. George Padgett, Dr. Ken Schwartz, and Dr. Doug Estry provided advice, encouragement and constructive criticism which helped keep the project moving forward and me on the right track. My sincere thanks to Mr. and Mrs. Kovalic for their support in the project by providing normal and heterozygote dogs for the study.

Even though I have dedicated this dissertation to my wife and daughter, I would like to say that I am especially grateful to my loving wife, Debbie, who had tremendous patience and understanding throughout my program. She deserves much credit for this degree.

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LIST OF ABBREVIATIONS

ADP................Adenosine Diphosphate

ATP................Adenosine Triphosphate

BHT................Basset Hound Hereditary Thrombopathy or Basset Hound Thrombopathic

BSS................Bernard-Soulier Syndrome

CBR................Coomassie Blue R

CIE................Crossed Immunoelectrophoresis

c-AMP..............Cyclic Adenosine Monophosphate

GP.................Glycoprotein

GT.................Glanzmann's thrombasthenia

NR-R...............Nonreduced-Reduced

PRP................Platelet Rich Plasma

SDS-PAGE...........Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

VWD................von Willebrand's Disease

VWF................von Willebrand's Factor

CHAPTER 1

INTRODUCTION

In normal hemostasis, the blood vessel wall, platelets, and plasma clotting factors all interact to arrest bleeding. A dysfunction of any of the three components can cause a hemorrhagic diathesis which may or may not be clinically evident until the hemostatic mechanism is stressed. Although they have not always been recognized as such, platelets now appear to play a central role in normal hemostasis. They adhere to injury sites, recruit more platelets by releasing their internal granules, and aggregate with other platelets to form the primary platelet plug, and in the course of events, they help to accelerate the normal coagulation cascade. Also, some of the released granule components and synthesized products aid in the hemostatic process due to their vasoactive nature. Due to their central role in hemostasis, platelets which are quantitatively or qualitatively abnormal can cause severe bleeding problems.

Qualitative platelet defects can be classified into defects of adhesion, release, or aggregation, and each type of defect can be investigated separately. Disorders of adhesion include Bernard-Soulier Syndrome (BSS) and von Willebrand's Disease (VWD). In BSS there is an absence of platelet membrane glycoprotein Ib (GP Ib) (1), and in VWD there is an absence of a plasma factor which is part of the

Factor VIII molecule called von Willebrand Factor (VWF)(2). It is now believed that VWF forms a bridge between GP Ib and the subendothelial tissue after an injury and is the molecular event responsible for adhesion (1). An absence or dysfunction of either GP Ib or VWF would result in defects in adhesion and a hemorrhagic diathesis.

Inherited defects in platelet release are probably quite rare and would include storage pool disease and cyclo-oxygenase deficiency. However, acquired release defects are common and are usually caused by aspirin and other non-steroidal anti-inflammatory agents which are irreversibly antagonistic to platelet membrane cyclo-oxygenase and thromboxane synthesis (3). Some other drugs may affect release by other mechanisms, but the effects are usually reversible.

Aggregation defects are recognized by an *in vitro* failure of platelets to aggregate in response to various stimuli. Perhaps the most common inherited aggregation defect in humans is Glanzmann's thrombasthenia (GT). In GT there is an absence or reduction of platelet membrane glycoproteins IIb and IIIa (GP IIb-IIIa) (4) which are normally present as a calcium-dependent heterodimer in the platelet membrane (5,6). Since GT platelets have both decreased GP IIb-IIIa and fibrinogen binding, the platelet membrane fibrinogen receptor is presumably located on the GP IIb-IIIa complex (7). It has long been known that fibrinogen and calcium are required for platelet aggregation, and

investigators now believe that the dimeric fibrinogen molecule forms a bridge between adjacent platelets by binding to GP IIb-IIIa which is exposed by stimulation with various aggregating agents (8). It follows then that defective or absent fibrinogen, absent or reduced GP IIb-IIIa, or abnormal exposure of the fibrinogen receptor would all be potential causes of aggregation defects.

An animal model with an inherited platelet aggregation defect has been identified and clinically the bleeding diathesis resembles the human disease Glanzmann's thrombasthenia (GT)(9,10). The defect was found in a colony of line-bred Basset Hounds and is named Basset Hound Hereditary Thrombopathy (BHT) (10). These Basset Hounds were found to have variably prolonged bleeding times, normal blood coagulation parameters, normal platelet numbers and morphology, normal whole blood clot retraction, but an abnormal platelet aggregation response to adenosine diphosphate (ADP) and collagen (10). The aggregation defect was similar to that seen with thrombasthenic platelets but the presence of normal clot retraction was a factor which immediately distinguished BHT from GT. It was decided at this point to investigate the possibility of storage pool deficiency, platelet membrane glycoprotein abnormalities, and platelet membrane fibrinogen binding in an attempt to ascertain the molecular defect responsible for the aggregation defect. The following chapters (11-13) are reprints of articles submitted and/or published which

attempt to systematically define the pathogenesis of BHT.

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CHAPTER 2

ABNORMAL RELEASE OF STORAGE POOL ADENINE NUCLEOTIDES FROM PLATELETS OF DOGS AFFECTED WITH BASSET HOUND HEREDITARY THROMBOPATHY

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ABSTRACT

Platelets from dogs affected with Basset Hound Hereditary Thrombopathy (BHT), have a thrombasthenia-like aggregation defect but release storage pool ATP in quantities not significantly different from normal controls or BHT heterozygotes when stimulated with 1×10^{-5}M ADP and 0.22 U/ml thrombin. However, the release occurs so rapidly in the BHT platelets stimulated with ADP that it is complete in approximately one-sixth of the time required for release from normal control and heterozygote platelets. Sequential electron micrographs reveal early release of BHT dense body constituents 30 seconds after stimulation with 1×10^{-5}M ADP while resting BHT morphology is indistinguishable from normal control animals.

INTRODUCTION

Recent characterization of a group of closely related Basset Hounds experiencing hemorrhagic diathesis has revealed a defect in intrinsic primary platelet aggregation (1). This syndrome has been labeled Basset Hound Hereditary Thrombopathy (BHT), and has been shown to be inherited as an autosomal recessive trait (1). Data on various hemostatic parameters revealed variably prolonged bleeding times, a normal blood coagulation mechanism, normal platelet numbers and morphology, normal whole blood clot retraction, and a defective platelet aggregation response to adenosine diphosphate (ADP) (1,2). Except for normal clot retraction, this syndrome closely resembles thrombasthenia in humans in which there is absent or reduced clot retraction (3). In general, failure of platelets to arrest bleeding may be attributed to defects

Keywords: platelets; adenine nucleotides; dense body release; thrombasthenia-like thrombopathia.

in adherence, aggregation, or release of platelet storage pool granule constituents (4), all of which require interaction between plasma and platelet components. In BHT, the plasma component appears to be normal (1,2) but previous data suggested a failure of ADP induced aggregation. The analysis of platelet aggregation data in BHT dogs revealed an inordinately large and prolonged shape change and the loss of dense body granules on electron micrographs; further, mixtures of normal and BHT platelets appeared to have enhanced aggregation.

This paper documents a difference of platelet nucleotide release in BHT and compares it to normal and heterozygote dogs. Also included is a sequential electron microscopic study of BHT and normal dog platelets after stimulation with ADP.

MATERIALS AND METHODS

The experimental subjects were grouped into three categories. The BHT or affected group consisted of four Basset Hounds previously studied (1) which had no platelet aggregation in response to 0.2×10^{-5}M ADP, a concentration that caused maximal aggregation in normal and carrier dogs. Normal controls consisted of two Basset Hounds proven to be normal when tested for platelet aggregation, and two unrelated Golden Retrievers previously characterized (1). The carrier group consisted of four heterozygote Basset Hounds that had produced BHT affected offspring, but exhibited normal platelet aggregation.

Blood for platelet aggregation was collected in trisodium citrate, platelet rich plasma (PRP) prepared, platelet counts performed, and platelet counts standardized to 3×10^{5} platelets/ul as described previously (1). Platelet aggregation studies were performed within 4 hours of blood collection.

Storage pool adenine nucleotide release was monitored using the Lumi-Aggregometer (Chrono-Log Corp., Havertown, Pa) according to Charo et al. (5). The Lumi-Aggregometer uses a firefly luciferin-luciferase system to detect released adenosine triphosphate (ATP), and simultaneously measures release of ATP and platelet aggregation. Monitoring was performed at 37°C with a teflon-coated stir bar rotating at 900 rpm within the sample. Results were recorded on a dual-pen Houston Omniscribe Chart Recorder (Houston Instrument Division of Bausch & Lombe, Inc., Austin, TX). Aggregation cuvettes contained 0.45 ml. of PRP (3×10^{5} platelets/ul) and 0.05 ml of Chrono-Lume^{R} No. 395 luciferin-luciferase reagent (Chrono-Log Corp., Havertown, PA) added to the PRP immediately prior to incubation at 37°C. After temperature equilibration for 2 minutes with stirring, either ADP or thrombin was added to a final concentration of 1×10^{-5}M and 0.22 U/ml respectively (ADP was obtained from Sigma Chemical Company, St. Louis, MO; thrombin was a generous gift from Dr. Houria Hassouna, Department of Pathology, Michigan State University prep #174; Activity=5664 u/ml; 99.6% Activity). Aggregation and release were monitored until the luminescence caused by the released storage pool ATP had started to subside. At this point a standard solution of ATP (final concentration of 4×10^{-6}M) was added. The amount of ATP released from the platelets was then calculated by using the peak luminescence point from both the platelet release and the addition of the standard concentration of ATP. The standard ATP solution was run with all samples to avoid slight changes due to reagent and/or platelet degradation, and all reagents were kept on ice until added to the aggregometer cuvettes. The time from injection of the aggregating agent to maximum luminescence was measured directly from the chart using the time increments and running the recorder at a speed of one inch per minute.

Platelets from each of the control animals were used to determine the optimum concentration of ADP to be used to stimulate release from platelet granules. This was performed by measuring platelet ATP released in response to five concentrations of ADP; 0.1×10^{-5}M, 0.2×10^{-5}M, 0.6×10^{-5}M, 1.0×10^{-5}M, and 2.0×10^{-5}M. Since maximum release of storage pool ATP was desired, the concentration beyond which there was no significant increase in ATP release was chosen as optimum.

Platelets used for electron microscopic studies were stirred at 900 rpm for two minutes at 37°C and then ADP (1.0×10^{-5}M final concentration) was added to all samples except the resting samples from BHT and normal control dogs. At various times after addition of ADP, 0.5 ml of 0.1% glutaraldehyde solution was added and the sample allowed to stir for an additional one minute. The specimens were then centrifuged for 15 seconds at 12,800 X g, the supernatant discarded, and 1.0 ml of 3% glutaraldehyde added. The specimens were capped and allowed to fix for a minimum of thirty minutes at which time they were transferred to a 3% buffered glutaraldehyde solution and processed for electron microscopy as described by Mattson, et al. (6). This procedure was followed for unstimulated platelets, and for samples at 30, 100, and 200 seconds after addition of ADP to BHT and normal control platelets.

RESULTS

Effect of ADP concentration on the quantity of ATP released. Since the objective of this investigation was to determine the availability and release of storage pool adenine nucleotide in BHT platelets, a concentration of ADP causing maximal release was desired. As can be seen in Fig. 1, a significant increase in the amount of ATP released occurs when the ADP concentration

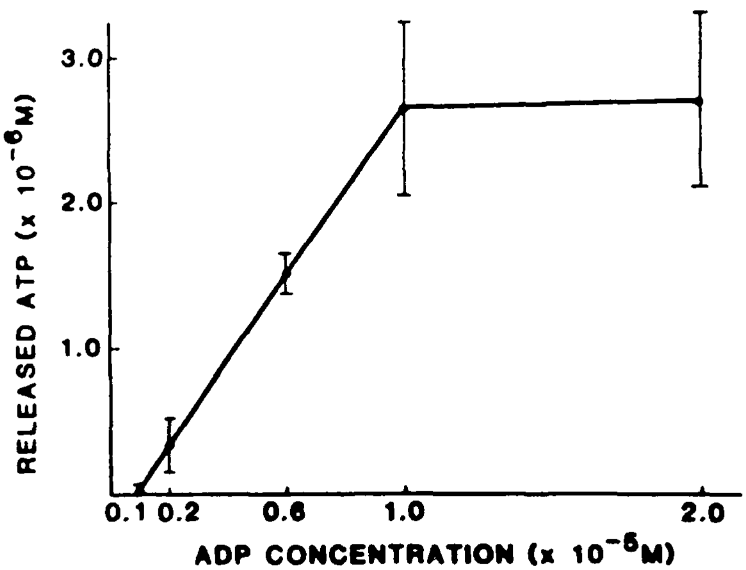


FIG. 1

Platelet ATP release during aggregation from four normal dogs (duplicate determinations) in response to varying concentrations of ADP used as the aggregating agent.

is raised from 0.1×10^{-5}M to 1.0×10^{-5}M, but the amount of ATP released does not increase significantly at higher concentrations of ADP. Therefore, 1.0×10^{-5}M ADP was used to stimulate platelet aggregation and release. Varying the concentration of thrombin from 0.22 - 0.44 U/ml did not cause significant differences in the amount of ATP released, however thrombin concentrations below 0.22 u/ml showed significant specimen to specimen variation in ATP release.

<u>Potentiation of aggregation by Chrono-Lume[R].</u> While monitoring aggregation, an apparent potentiation of aggregation was observed in samples containing Chrono-Lume[R] and stimulated with ADP similar to that observed by Mehta, et al. (7)(Fig.2).

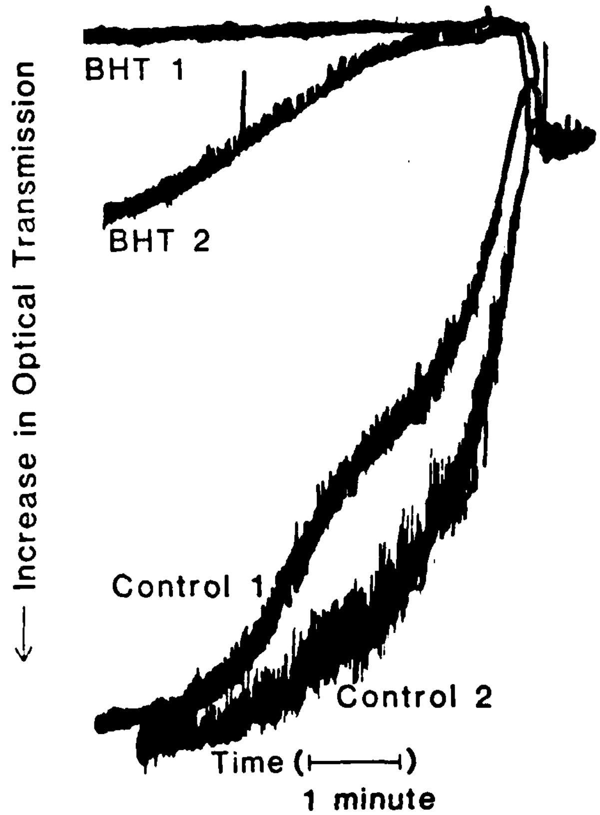


FIG. 2

Platelet aggregation tracings in response to 1.0×10^{-5}M ADP. Normal dog platelets were collected from two normal Basset Hounds and two unrelated Golden Retrievers. BHT platelets were collected from four BHT affected Basset Hounds. In all cases the slope and extent of aggregation were increased in samples containing Chrono-Lume[R] (BHT 2 and Control 2).

<u>Release of ATP and time to maximum luminescence.</u> The data from these measurements are shown in Table 1 and Fig 3. It can be observed from the data in Table 1 that the quantity of ATP that is released and the time to maximum luminescence in response to thrombin are not significantly different when all three groups are compared by the student T test ($p < .001$). This rules out a storage pool abnormality as a cause for the aggregation defect. However, the time to maximum luminescence in response to ADP is significantly different in the BHT group when compared to normal and proven heterozygous dogs ($p < .001$).

BHT platelets reach maximum luminescence, indicating maximum release of storage pool ATP, in approximately one sixth of the time required for normal platelets.

TABLE 1

Platelet ATP release and time to maximal luminescence in BHT, normal, or dogs heterozygous for BHT(1)

	Normal		Heterozygous		BHT	
	ADP*	Thrombin†	ADP *	Thrombin†	ADP*	Thrombin†
RELEASED ATP ($\times 10^{-6}$M) MEAN$\pm$SD**	2.64 $\pm$0.60	2.88 $\pm$0.79	2.94 $\pm$0.49	2.47 $\pm$0.35	3.00 $\pm$0.42	2.75 $\pm$0.85
TIME TO MAXIMUM LUMINESCENCE (MINUTES) MEAN$\pm$SD**	2.64 $\pm$0.34	3.12 $\pm$0.47	2.20 $\pm$0.44	3.87 $\pm$0.47	0.41 $\pm$0.06	2.97 $\pm$0.64

(1)*1×10^{-5}M; † 0.22 u/ml; ** n = 10

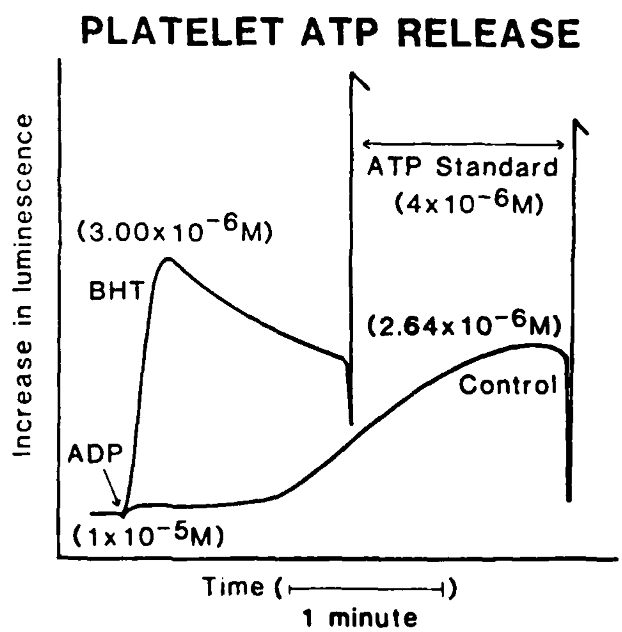


FIG. 3

Platelet ATP release in response to 1×10^{-5}M ADP. Release of ATP occurs significantly faster from BHT platelets than from controls. Numbers in parentheses are peak values representing the mean values from BHT and control platelets (Table 1).

Electron microscopic findings. Sequential electron micrographs of BHT and normal platelets taken at 30, 100, and 200 seconds after stimulation with ADP, morphologically support early release of dense granules from BHT platelets. Unstimulated (resting) platelets appear similar in both groups. At 30 seconds after stimulation the BHT platelets display shape change, centralization of granules, lack of aggregation, and absence of dense granules. In contrast, the normal platelets show shape change, but no centralization of granules, no microtubule formation, presence of dense granules, and partial aggregation. The 100 and 200 second BHT samples showed a lack of platelet aggregation. With the 200 second sample, an apparent dissociation of microtubules was also noted. The 100 and 200 second samples rrom controls displayed significant platelet aggregation and dense granules were still present in a few platelets.

DISCUSSION

Normal hemostasis is accomplished through a complex set of interacting factors that involve platelets, the vascular wall and the coagulation factors (8). The platelet's hemostatic role depends on the ability to change shape, adhere to subendothelial tissue, release storage pool granule constituents, and to aggregate into viable and stable plugs (9,10). To do this, there must be a functional receptor on the platelet membrane, and the stimulated receptor must transmit to the platelet interior. Then microtubule mediated concentration and release of platelet granules occur. Finally, surface fibrinogen receptors are exposed and react with fibrinogen, followed by platelet-platelet interaction and evolution of a stable plug (11-13).

This investigation has documented that BHT platelets change shape in response to an aggregation stimulus, and also release normal amounts of storage pool granule ATP. Further, citrated resting BHT platelets appear ultrastructurally normal on transmission electron microscopy. Previous studies have documented normal BHT plasma factors and platelet adhesion (1,2).

Although ADP stimulated BHT platelets change shape and release normal amounts of storage pool granule constituents, the rate of release is abnormal and they do not aggregate. Thrombin stimulated BHT platelets, however, release in a normal fashion but still do not aggregate. Lack of aggregation may point to a defect of the fibrinogen receptor, which has been shown by several investigators to be a calcium dependent complex of two specific glycoproteins; glycoproteins IIB and IIIa (GP IIb-IIIa) (14,15) or to defective fibrinogen receptor exposure. The inability of BHT platelets to bind fibrinogen or to expose fibrinogen receptors may, in some way, be related to rapid ATP release after ADP stimulation. One may be the cause of or be involved in causing the other. A possible mediator in both of these reactions could be Ca^{++}. It has been shown that ADP induced platelet activation increases surface bound Ca^{++} and the exchange rate of Ca^{++} into the intracellular pool without increasing the amount of intracellular Ca^{++} (16). An abnormality in either the rate or quantity of calcium exchange might explain the rapid ATP release and aggregation defects in BHT. The fact that BHT platelets support normal clot retraction but fail to aggregate seems to be an enigma. However, there are distinct receptors for fibrinogen, required for aggregation, and for fibrin, required for clot retraction (17).

Lastly, platelet ectosialyltransferase activity has been shown to be involved in cell adhesion and platelet aggregation (18). The receptor for the enzyme has been shown to be glycoprotein IIb (19). Altered platelet ecto-

sialyltransferase activity could be responsible for defective aggregation in BHT, since GP IIb is part of the fibrinogen receptor complex and the receptor for the enzyme.

Further understanding of this platelet defect will require specific study of membrane fibrinogen receptors, fibrinogen binding, calcium binding, and ectosialyltransferase activity. By using this animal model to examine platelet fibrinogen interaction, calcium involvement, and aggregation, the importance of platelet involvement in thrombosis may be extended.

LEGENDS FOR ELECTRON MICROGRAPHS

Figure 4. Citrated resting platelets from a normal control dog(X 9000). Platelets display normal discoid shape and evenly dispersed dense granules (closed arrow).

Figure 5. Citrated resting platelets from a dog affected with BHT(X 9000). These platelets are similar to the normal platelets in Fig. 4 and also show discoid shape and evenly distributed dense granules (closed arrow).

Figure 6. Normal dog platelets (X 9000) 30 seconds after stimulation by ADP show shape change (pseudopodia, open arrows), no centralization of granules, no microtubule formation, partial aggregation, and dense granules are still present (closed arrow).

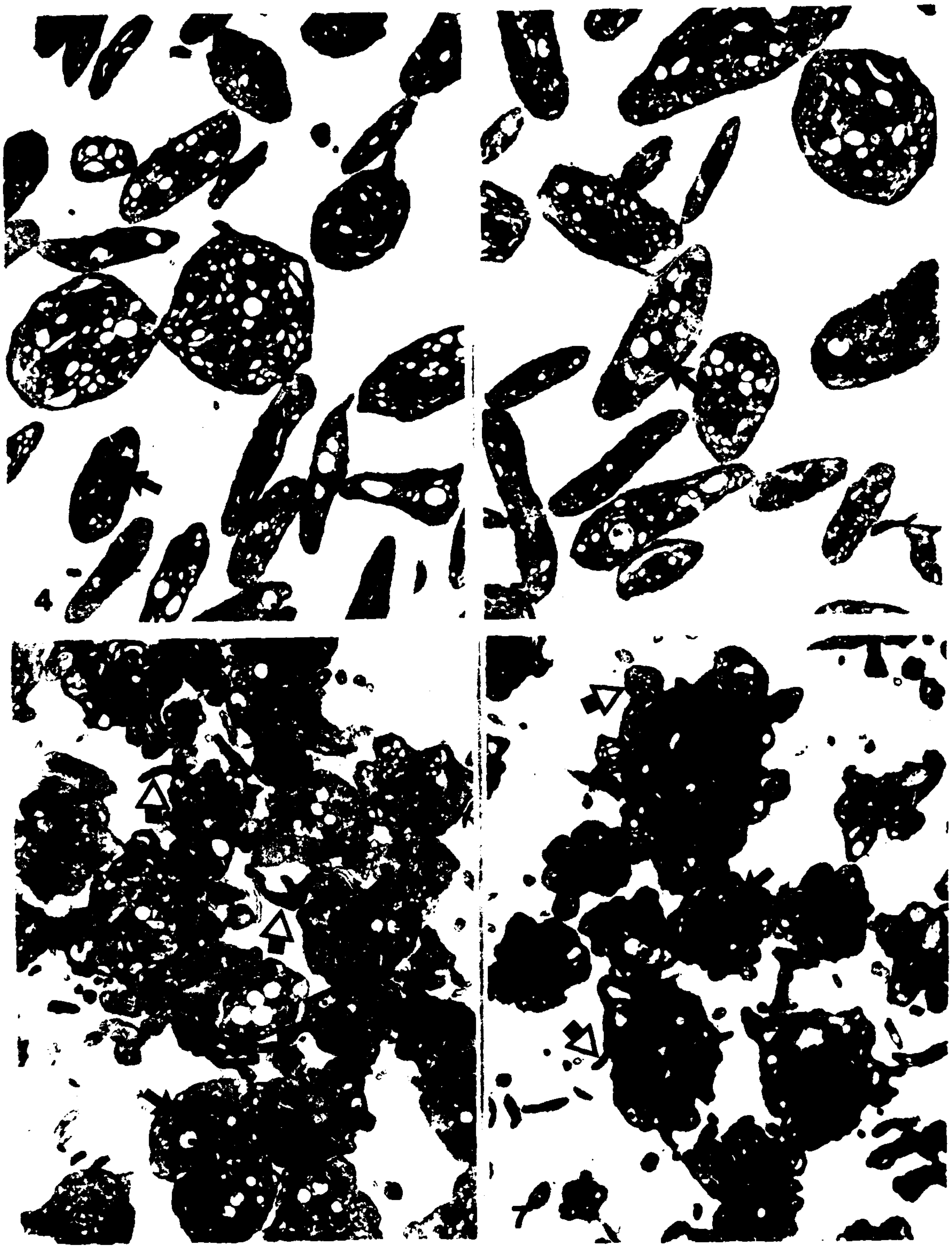
Figure 7. BHT affected platelets (X 9000) 30 seconds after stimulation with ADP show shape change (pseudopodia, open arrows), centralization of granules by microtubules (closed arrow), lack of aggregation, and no dense granules which is in agreement with release data.

Figure 8. Normal dog platelets (X 9000) 100 seconds after stimulation with ADP show significant aggregation and the presence of non-centralized dense granules (closed arrows).

Figure 9. BHT platelets (X 9000) 100 seconds after stimulation with ADP are similar to the 30 second BHT sample with definite centralization of granules by microtubules, no dense granules, no aggregation, and shape change (pseudopodia, open arrows).

Figure 10. Normal dog platelets (X 9000) 200 seconds after stimulation with ADP show total aggregation and the presence of fewer dense granules (closed arrow) than previous samples (Figures 6 and 8).

Figure 11. BHT platelets (X 9000) 200 seconds after stimulation with ADP show no aggregation and an apparent dissociation of microtubules. Pseudopodia are still evident (open arrow) and there are no dense granules present.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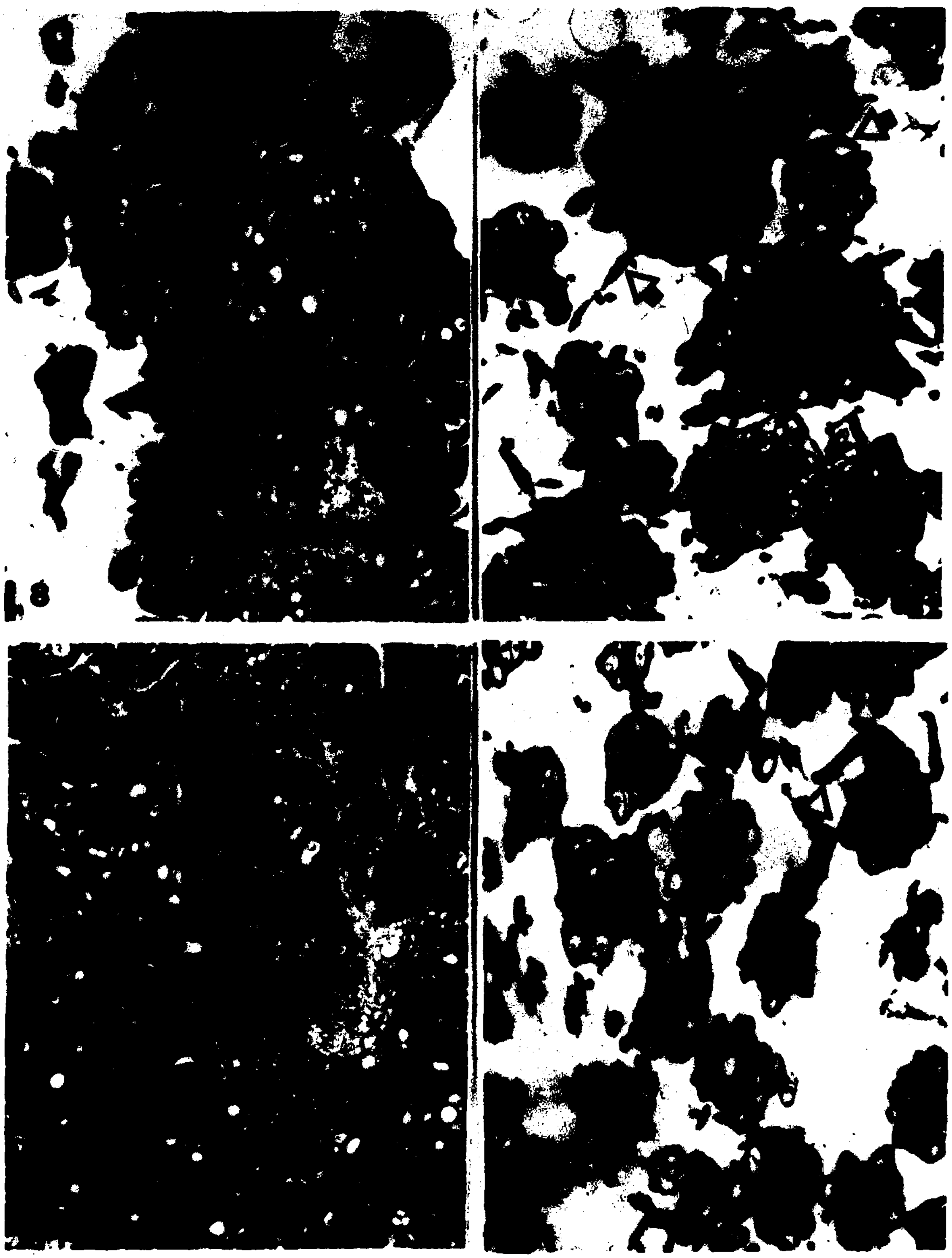

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CHAPTER 3

TWO-DIMENSIONAL ELECTROPHORETIC STUDIES OF PLATELETS FROM DOGS AFFECTED WITH BASSET HOUND HEREDITARY THROMBOPATHY: A THROMBASTHENIA-LIKE AGGREGATION DEFECT

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ABSTRACT

Because of a thrombasthenia-like platelet aggregation defect, platelets from dogs affected with Basset Hound Hereditary Thrombopathy were compared to normal control dog platelets by three different techniques in order to assess platelet membrane glycoprotein content. Crossed immunoelectrophoresis (CIE), two-dimensional nonreduced-reduced electrophoresis (NR-R), and O'Farrell two-dimensional electrophoresis were used for the assays. CIE and NR-R gels detected no differences between affected Basset Hound and control dog platelets. Gels run by the O'Farrell technique detected no differences in glycoprotein/protein content, however, there appear to be several constituents missing from BHT affected dog platelet samples. The missing components appear to be either lipids or sialoglycoproteins as they were detectable by silver staining but not by Coomassie Blue staining.

INTRODUCTION

Previous studies on platelets from a group of closely related Basset Hounds revealed a thrombasthenia-like intrinsic

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Key Words: Platelets, platelet membranes, platelet glycoproteins, thrombasthenia-like.

platelet aggregation defect which was named Basset Hound Hereditary Thombopathy (BHT)(1,2), and has been shown to be inherited as an autosomal recessive trait (2). Other studies on BHT affected dogs revealed normal intrinsic and extrinsic coagulation mechanisms, variably prolonged template bleeding times, normal platelet count and morphology, normal clot retraction, and defective platelet aggregation responses to adenosine diphosphate (ADP) (2). More recently, studies on release of storage pool adenosine triphosphate (ATP) detected normal quantities of ATP being released in response to ADP and thrombin, but in response to ADP (and not thrombin), the release occurred so rapidly that it was complete in about one-sixth of the time required for release to occur from normal control dog platelets (3).

Several plasma and platelet constituents have been shown to be required for normal platelet aggregation. Amoung these are divalent calcium ions (4,5), fibrinogen (6,7), and platelet membrane glycoproteins IIb and IIIa (8,9). It has been shown by various methods that the calcium dependent glycoprotein IIb-IIIa (GP IIb-IIIa) complex is the receptor for fibrinogen on the platelet membrane (10-12). In Glanzmann's Thrombasthenia, a disease in which several platelet membrane glycoproteins are reduced or absent, platelet aggregation and fibrinogen binding are severely affected, being either totally absent or greatly reduced (13,14).

Because of the evidence that the complex of glycoproteins IIb and IIIa in platelet membranes mediates platelet aggregation and that an absence or reduction of the complex results in defective platelet aggregation, platelets from dogs affected with BHT were compared to normal control dog platelets as to their platelet protein and glycoprotein composition. Two- dimensional nonreduced-reduced electrophoresis (NR-R), crossed immuno-electrophoresis (CIE), and two-dimensional O'Farrell sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) techniques were used to determine the presense of platelet membrane abnormalities.

MATERIALS AND METHODS

All reagents, buffers, and chemicals used were of the highest purity. SDS-PAGE and staining reagents were obtained from Bio-Rad (Richmond, California). GEL-BOND, GEL-BOND PAG, and agarose were obtained from FMC Corporation (Rockland, Maine), electrophoresis equipment used was a Bio-Rad PROTEAN II system (Bio-Rad, Richmond, Calif.) and an LKB Multiphor system (LKB, Stockholm, Sweden). AP-2, a monoclonal antibody to the GP IIb-IIIa complex was acquired from Dr. Thomas Kunicki (Milwaukee, Wisconsin), and an anti-human platelet antibody was obtained from Accurate Chemical & Scientific Corporation (Westbury, NY).

Blood was collected by jugular venipuncture and platelet rich plasma prepared from BHT and normal control dogs as previously described (2). Platelet aggregation was performed on a LUMI-AGGREGOMETER (Chronolog, Haverstown, PA) as described previously (2). Washed platelets for electrophoresis procedures were prepared as described by Kristopeit and Kunicki (15) and Nurden, et al (16). After the final wash, the platelets were resuspended in a volume of Tris-glycine buffer (15) sufficient to give a platelet count of 5-6 X 10^9 platelets/ml. Platelets for nonreduced-reduced two-dimensional electrophoresis (NR-R) were solubilized with SDS in the presence of 10mM N-ethyl maleamide (16). Platelets for O'Farrell two - dimensional electrophoresis were solubilized with SDS in the presence of 2-mercaptoethanol according to Anderson et al (17). The specimens were incubated at 100°C for 5 minutes, aliquoted, and stored at -80°C until assayed. Platelets for crossed immunoelectrophoresis were radiolabeled and solubilized according to Phillips, et al (18).

Electrophoresis of the solubilized platelet samples for NR-R gels was performed according to Nurden, et al (16) with modifications. Tubes for first dimension gels were 3.0mm (ID) X 125mm and 200 ug samples were electrophoresed for 4-5 hours at 100 volts until the bromophenol blue front was about 1/2 cm from the bottom of the tube. The gels were removed from the tubes and placed in 15 ml reducing buffer (16), and placed on a rocker for one hour, after which they were annealed to the second dimension slab gels with 1% agarose.Electrophoresis by the O'Farrell method uses isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. First dimension O'Farrell gels and buffers were prepared according to O'Farrell (19) using an ampholyte pH range of 3-10 (Pharmolytes, Sigma Chemical Company, St. Louis, MO) and 2.0mm (ID) X 125mm tubes. The samples were electrophoresed for 16 hours at 400 volts and then one additional hour at 600 volts. The gels were then placed in equilibration buffer (2% SDS, 5% 2-ME, 10% glycerol, 50mM Tris) with rocking for two hours after which they were annealed to second dimension gels with 1% agarose. Slab gels for second dimension electrophoresis were 16 X 16 cm square and 1.5 mm thick and were cast using GEL-BOND PAG as a backing. Buffer for second dimension electrophoresis was 0.05 M Tris, 0.384 M glycine, and 0.1% w/v SDS for both NR-R and O'Farrell gels. Second dimension electrophoresis was performed at 40 volts for 16 hours. Staining of the gels was with Coomassie Blue R (CBR) for NR-R gels and silver - coomassie blue double staining according to Dzandu, et al (20) for O'Farrell gels.

Crossed immunoelectrophoresis was performed according to Kunicki, et al (10). Two antibodies were used; AP-2 which has been characterized as specific for the GP IIb-IIIa complex by Pidard, et al (21), and an anti-human platelet antibody. Autoradiographs were performed using Kodak XRP-1 film (Kodak, Rochester, NY) and were exposed for 36 hours at -80°C.

RESULTS

Platelet aggregation. Aggregation tracings of BHT and control dog platelets in response to 1 X 10^{-5}M ADP are shown in figure 1. Control dog platelets displayed a normal response to ADP, however, BHT dog platelets responded only by changing shape and no aggregation was noted. Increasing the concentration of ADP had no effect on BHT platelets (data not shown). Further discussion of the platelet aggregation abnormalities in BHT can be found in previous articles (2,3).

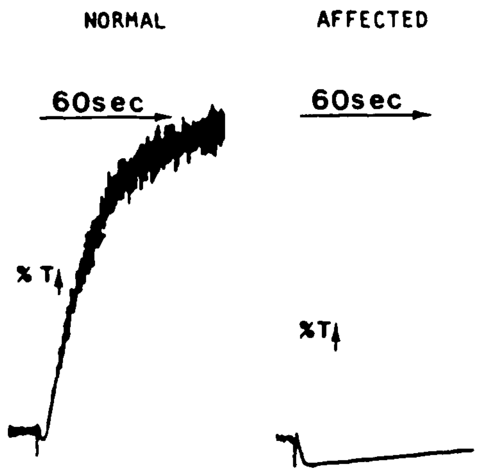

FIG 1

Normal and BHT platelet aggregation tracings in response to 1.0 X 10^{-5}M ADP. The BHT (Affected) platelet aggregation response shows only a decrease in percent transmission indicative of platelet shape change.

Crossed immunoelectrophoresis. Figure 2 demonstrates precipitin arcs detected when solubilized platelets were electrophoresed and autoradiographed using both AP-2 and an anti-human platelet antibody. The major precipitin arc seen in the gels is represented by the glycoprotein IIb-IIIa (GP IIb-IIIa) complex. Using AP-2, only GP IIb-IIIa is stained and autoradiographed(10). Dog platelets showed little antigen cross-reactivity with the anti- human platelet antibody with some variability in the number of precipitin arcs that are detected by CBR staining. However, the major platelet membrane glycoprotein antigen, the GP IIb-IIIa complex, is precipitated in all samples tested and appears normal in charge and quantity using both AP-2 and an anti-human platelet antibody.

Nonreduced-reduced two-dimensional gels. Figure 3 shows CBR

FIG 2

Crossed immunoelectrophoresis gels of radiolabeled, Triton X-100 solubilized platelets stained with Coomassie Blue R (A,B,C,D) and corresponding autoradiographs (E,F,G,H). Normal dog (A,E) and BHT platelets (B,F) using an anti-human platelet antibody. Normal dog (C,G) and BHT platelets (D,H) using AP-2, a monoclonal antibody specific for the GP IIb-IIIa complex. The gels show no differences between normal and BHT platelets.

FIG 3

Nonreduced-reduced two dimensional electrophoresis gels of SDS solubilized normal and BHT platelets stained with Coomassie Blue R. Several components have been labeled; Thrombospondin (Tsp), fibrinogen (Fbg), actin (Ac), and glycoproteins IIb, IIIa, and Ib (IIb, IIIa, and Ib). The gels revealed no differences between normal and BHT platelets by this technique.

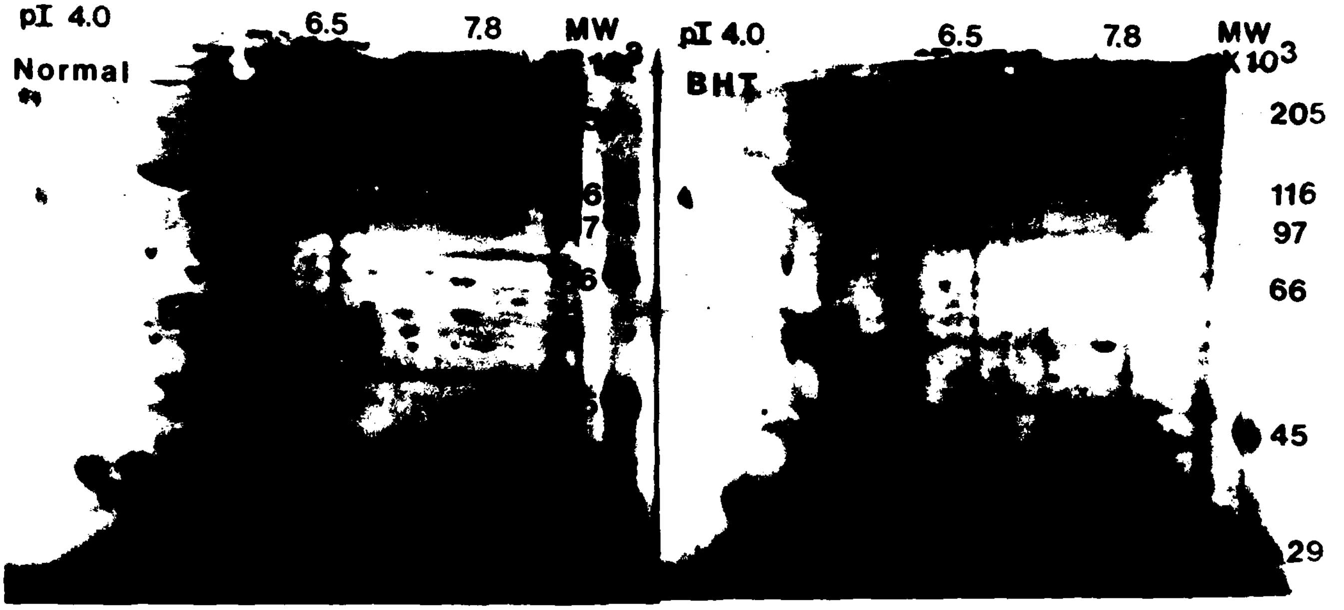

FIG 4

O'Farrell two-dimensional gels of SDS solubilized normal and BHT platelets stained by a Coomassie Blue R - silver double staining technique. The gels revealed no differences in the glycoprotein/protein content (Coomassie Blue staining spots) however, they did show apparent differences in that there are several missing components in the BHT sample (surrounded by arrows). These components stained yellow in the normal sample which indicates that they are possibly lipid or sialoglycoprotein in nature.

stained NR-R gels from normal and BHT affected dogs. Again, GP's IIb and IIIa are present in normal quantities as are glycoprotein Ib, platelet fibrinogen, thrombospondin, and other membrane and intracellular constituents. The NR-R gels showed complete homology with human specimens as NR-R gels of solubilized human platelets were indistinguishable from those of the dog samples (personal observation).

O'Farrell two-dimensional gels. Representative gels from normal control and BHT dog platelets are shown in Figure 4. The gels were stained by a silver-CBR double staining technique (20). There are no apparent differences between between normal and BHT platelets with respect to protein/glycoprotein content (Coomassie Blue R staining spots). The area of the gels surrounded by arrows shows several spots missing in the BHT sample when compared to the normal control sample. With the double staining technique these spots were yellow-brown in color and according to Dzandu, et al (20) they are probably lipids. All the CBR stained spots appeared identical in number and location when comparing normal

staining intensity which would indicate differences in protein concentration between samples. Every attempt was made to use 100ug protein samples, however, minor errors in protein measurement or pipetting cannot be ruled out. The missing spots in the BHT sample do not appear to be due to variations in concentration since they are completely absent rather than less intensely stained. Also, the silver staining technique is several times more sensitive than CBR staining.

DISCUSSION

It is now well recognized that some major events in the hemostatic process are mediated by platelet membrane glycoproteins. By studying platelets from patients with Bernard-Soulier Syndrome, glycoprotein Ib has been shown to be responsible for platlet adherence to subendothelium , since Bernard-Soulier platelets are deficient in glycoprotein Ib and do not adhere to exposed subendothelium (13,22). However, these platelets did show normal aggregation responses to ADP and collagen (22). Another inherited bleeding disorder, Glanzmann's Thrombasthenia, shows absent platelet aggregation responses to ADP, thrombin, and collagen, and is deficient in platelet membrane glycoproteins IIb and IIIa (14). Subsequent to these reports, the GP IIb-IIIa complex has been shown to be the calcium dependent platelet fibrinogen receptor which is necessary for normal platelet aggregation (8-12).

BHT resembles Glanzmann's Thrombasthenia in that there is an absence of platelet aggregation in reponse to various aggregating agents, but it is dissimilar in that there is normal clot retraction in BHT (2). Investigation of the BHT platelet membrane glycoproteins was indicated, however, due to the absence of platlet agrregation response. The presence of an apparently normal GP IIb-IIIa complex as demonstrated by CIE and NR-R gels rules out the possibility of BHT being an animal homologue of thrombasthenia, but, the possibility remains that the GP IIb-IIIa complex may be functionally abnormal. O'Farrell two- dimensional electrophoretic gels could theoretically demonstrate abnormalities in platelet composition if they resulted in changes in isoelectric point and/or molecular weight. Over a pH range large enough to encompass the majority of platelet components, however, the abnormalities would have to be gross enough to cause a relatively large change in the isoelectric point or molecular weight. Therefore, minor abnormalities could go undetected in this procedure. The comparison of BHT and normal control dog solubilized platelets by the O'Farrell technique did not reveal differences in glycoprotein and/or protein content, as was also evidenced by CIE and NR-R gels. However, O'Farrell gels did reveal some apparent lipid abnormalities, with several spots missing from the BHT sample. Further studies of BHT platelet membrane lipid, phospholipid, and glycolipid contents by more analytical techniques will be required to establish if an actual

lipid abnormality exists in BHT platelet membranes. Lipid differences, if present, could affect platelet membrane reorganization or fluidity or membrane receptor movement after stimulation which is essential to normal platelet function. Membrane reorganization may be essential for exposure of fibrinogen and other membrane receptors, and improper reorganization could adversely affect agonist induced platelet aggregation. Studies of agonist induced fibrinogen binding to BHT platelets is imperative in order to establish if GP IIb-IIIa, shown by electrophoretic techniques to be quantitatively normal, is functionally normal in BHT and is capable of binding fibrinogen. Fibrinogen binding studies are currently in progress.

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CHAPTER 4

FIBRINOGEN BINDING TO PLATELETS FROM DOGS WITH BASSET HOUND HEREDITARY THROMBOPATHY: A THROMBASTHENIA-LIKE AGGREGATION DEFECT

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Running Title: BHT Fibrinogen Binding

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ABSTRACT

Platelets from dogs with Basset Hound Hereditary Thrombopathy (BHT) display a thrombasthenia-like aggregation defect but have been shown to have normal amounts of platelet membrane glycoproteins IIb and IIIa (GP IIb-IIIa). The presence of normal quantities of GP IIb-IIIa, however, did not rule out the possibility of a functionally abnormal glycoprotein complex which would be unable to bind radiolabeled fibrinogen. Therefore, fibrinogen binding in BHT platelets was evaluated. Fibrinogen preparations from BHT and normal control dogs, as well as a human fibrinogen preparation were used. Platelets from BHT and normal dogs were activated with 1 X 10^{-5}M ADP in the presence of ^{125}I-labeled fibrinogen and the surface bound radioactivity was quantitated. For all fibrinogen preparations, the amount of fibrinogen bound by BHT platelets was not significantly different than that bound by normal dog platelets. BHT platelets bound 23,972 $\pm$ 3612 and normal dog platelets bound 23,033 $\pm$ 3971 molecules of fibrinogen per platelet. The quantitatively normal GP IIb- IIIa complex binds fibrinogen in normal amounts and does not seem to be the abnormality responsible for the aggregation defect in BHT platelets. The results show that the binding of fibrinogen is normal and that other factors, such as receptor mobility may help to explain the abnormal aggregation of BHT platelets.

INTRODUCTION

Central to the normal hemostatic process is the aggregation of platelets at the injury site. It has long been recognized that certain plasma factors, especially calcium and fibrinogen, are required for platelet aggregation to occur (1,2). Afibrinogenemic individuals have prolonged bleeding times (3), and platelets from individuals with Glanzmann's Thrombasthenia do not aggregate (4) or adsorb fibrinogen (5,6) which suggests that during clot formation fibrinogen and platelets are involved in specific interactions. Studies have shown that ADP induces the exposure of specific and saturable fibrinogen receptors on the platelet surface and that fibrinogen binding and platelet aggregability are closely correlated (7,8), with some studies indicating that the dimeric fibrinogen molecule may actually form a bridge between receptors on adjacent platelets (9).

Platelets from Glanzmann's thrombasthenia patients are characterized by a severely reduced fibrinogen binding capacity, as well as an inability to aggregate in response to ADP (6,9). One of the major defects in this disorder is the absence or reduction of platelet membrane glycoproteins IIb and IIIa (GP IIb- IIIa)(10-12). Evidence has accumulated that the calcium dependent GP IIb-IIIa complex is the platelet membrane receptor for fibrinogen and is responsible for platelet- platelet cohesion in ADP

antibodies specific for the GP IIb- IIIa complex, have been shown to cause inhibi- tion of both fibrinogen binding and platelet aggregation (18,19).

Basset Hound Hereditary Thrombopathy (BHT) is described as a thrombasthenia-like aggregation defect (20,21) with an abnormally rapid but quantitatively normal release of storage pool adenine nucleotides (22). In view of the thrombasthenia-like aggregation defect, the platelet membrane glycoprotein content was investigated and found to be normal (23). The study of radiolabeled fibrinogen binding to ADP activated BHT platelets was undertaken to ascertain if the quantitatively normal GP IIb-IIIa complex was functionally normal with respect to its fibrinogen binding capability.

METHODS

Platelets. Platelets were isolated and washed according to the method of Kunicki et al.(24). Briefly, six volumes of whole blood were drawn into one volume of acid-citrate-dextrose (ACD, NIH formula A) by non-traumatic jugular puncture as described by Bell et al. (21). Platelet rich plasma (PRP) was prepared by centrifugation at 1470 X g for 1.5 minutes. The centrifugation step was repeated two times and provided approximately 10 ml of PRP from 24 ml of blood. All manipulations were performed at room temperature. During washing, apyrase (2 U/ml) and prostaglandin E_1 (20 nmol/L)(Sigma Chemical Company, St. Louis, MO.) were used in the wash buffer to prevent inadvertent platelet activation. Platelets were resuspended to a concentration of 1 X 10^9 platelets/ml. Prepared platelets maintained their ability to bind fibrinogen for at least four hours.

FIBRINOGEN. Plasma fibrinogen was purified from BHT affected and normal control dogs according to Takeda (25). Human fibrinogen was obtained from Kabi Vitrum, Stockholm, Sweden. Purity of the fibrinogen preparations was checked by SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE). Clottability was determined to be approximately 95% before radioiodination and >90% after radioiodination. Fibrinogen preparations were radioiodinated with carrier free ^{125}I (Amersham, Arlington Heights, IL) using lacto-

peroxidase-conjugated beads (Enzymobeads, BioRad Laboratories, Richmond, CA) according to the manufacturer's instructions. Unbound ^{125}I was removed from the fibrinogen reaction mixture by gel filtration through Sephadex G-50. The specific activity of the fibrinogen preparations was determined to be approximately 100 cpm/ng. Labeled fibrinogen was used within one week and stored at $4^{o}C$.

FIBRINOGEN BINDING. Binding assays were performed as described by Kunicki, et. al. (24). Non-specific binding was determined and was subtracted to give specific binding. BHT and normal dog platelets were reacted in triplicate with each of the three fibrinogen preparations, i.e. BHT, normal, and human fibrinogen.

PLATELET AGGREGATION. Blood was collected and PRP prepared according to Bell et al. (21). Platelet aggregation studies were performed using a Lumi-Aggregometer (Chronolog Corp, Haverstown, PA). PRP was adjusted as necessary to a final platelet count of 300 X 10^3/ul with platelet poor plasma. ADP, to a final concentration of $1X10^{-5}$, was added to 0.45 ml PRP with mixing at 900 rpm and at $37^{o}C$. The chart recorder was run at 2.5 cm/min.

STATISTICS. The Students t Test was used to compare BHT platelets to normal dog platelets as to the number of fibrinogen molecules bound per platelet. Significance is specified as $p < .05$.

RESULTS

Platelet Aggregation. As seen in figure 1, the aggregation response of BHT platelets, when compared to platelets from normal dogs, is totally absent except for a decrease in percent transmission corresponding to platelet shape change. Increasing the concentration of ADP to $2X10^{-5}M$ had no effect on the aggregation response as has been previously reported (22).

Fibrinogen Binding. Three different purified fibrinogen preparations were used to exclude the possibility that there might be a fibrinogen abnormality that would affect binding to stimulated platelets. As seen in table 1, the mean values of number of molecules of fibrinogen bound per ADP activated platelet for each fibrinogen preparation, and the overall means for all preparations are not significantly different between BHT and normal dog platelets. The overall values, 23,972 ± 3612 molecules per platelet for BHT and 23,033 ± 3971 molecules per platelet for normal control dogs show that BHT platelets do in fact bind fibrinogen. Fibrinogen binding values for both test groups are comparable to those obtained by other investigators for human platelets (26,27). It is also interesting to note that human fibrinogen will bind to dog platelets with no apparent species specific differences in total binding. It appears that a quantitatively normal GP IIb-IIIa complex, as reported previously (23), is also functionally normal in

its fibrinogen binding capacity.

Competitive inhibition of ^{125}I-fibrinogen binding to ADP activated platelets by unlabeled fibrinogen is illustrated in figure 2. The total amount of fibrinogen in the reaction mixture was constant with only the percentage of labeled fibrinogen being varied. The linear relationship of the number of ^{125}I-fibrinogen molecules bound versus the percentage labeled fibrinogen in the reaction mixture established that the binding of fibrinogen to ADP activated dog platelets is specific as has been previously reported by Marguerie et al. for human platelets (28).

DISCUSSION

Fibrinogen is a cofactor in ADP- induced platelet aggregation (1-7), and its binding to specific receptors on the platelet membrane mediates platelet- platelet cohesion and aggregation (4,6,9,29). A four phase sequence for fibrinogen binding as proposed by Marguerie and Plow (29) involves platelet activation, induction of the fibrinogen receptor, reversible fibrinogen binding, and fibrinogen-platelet complex stabilization. Many agonists cause platelet activation, some by seemingly different pathways, but each seems to lead to exposure of fibrinogen receptors and fibrinogen binding. The mechanics of fibrinogen receptor exposure on platelets is somewhat of a controversy at present. Shattil et al.(30) reported that receptor exposure involves a conformational change in GP IIb-IIIa which exposes the fibrinogen binding site, while Coller (31) presents evidence for a membrane microenvironmental change which exposes the receptor.

Studies with platelets from patients with Bernard-Soulier and Glanzmann's thrombasthenia show that the calcium dependent membrane GP IIb-IIIa complex is the major platelet receptor for fibrinogen (4,6,9). Platelet activation by ADP is attributed to an agonist induced increase in cytoplasmic calcium ion concentration (32), with exposure of fibrinogen receptors being part of the overall response. These responses can be blocked by raising

the c-AMP levels in platelets (33,34).

BHT is characterized by an absence of a platelet aggregation response with an abnormally rapid release of normal quantities of storage pool adenine nucleotides (22). The aggregation tracings are similar to those observed with Glanzmann's thrombasthenic platelets (21,22). Investigation of the platelet membrane GP IIb-IIIa complex in BHT platelets revealed apparently normal quantities and complexing of the glycoproteins, in contrast to Glanzmann's platelets which show absent or reduced amounts of GPIIb-IIIa (9). Two obvious possibilities for the aggregation defect were that the GP IIb-IIIa complex was present in the membrane in normal amounts, but was unavailable to fibrinogen because of other defects which prevented its exposure after ADP stimulation or, that the receptor was unable to bind fibrinogen once it was exposed. Either of these alternatives would evidence themselves as absent or reduced fibrinogen binding. However, in this report we show that the number of fibrinogen molecules bound per platelet by BHT platelets is identical to that bound by normal dog platelets. Therefore, fibrinogen receptor exposure and binding must be normal in BHT. This suggests that fibrinogen binding is necessary for platelet aggregation but is not sufficient in and of itself for normal aggregation. This phenomenon was reported previously by Peerschke and Zucker (34), who observed that fixed ADP stimulated platelets bound fibrinogen but failed to

aggregate when shaken. Receptor movement within the membrane may also be essential for normal platelet aggregation, and simply binding fibrinogen is not enough to support aggregation. Fibrinogen receptor redistribution in platelets after activation has been reported by Loftus and Albrecht (35) and Estry et al. (36), who used fibrinogen-labeled colloidal-gold to directly visualize fibrinogen receptors on platelet membranes. Their experiments showed no fibrinogen binding in platelets immediately after contact which was followed by fibrinogen binding and redistribution with localization over the granulomere. Receptor movement would seem to be part of the normal course of events in platelet aggregation and abnormalities in membrane fluidity may have an antagonistic effect on platelet aggregation. It is interesting to note that an apparent lipid defect was observed in BHT platelets by two-dimensional O'Farrell electrophoretic techniques (23), but more analytical techniques are required to fully establish if a lipid defect is actually present. Another possible explanation for the defect may be an abnormal membrane glycoprotein-cytoskeletal protein interaction. Kalomiris and Coller (37) provide evidence to suspect that protein sulhydryl and disulfide groups may contribute to various platelet functions, especially those in which membrane related events influence cytoplasmic events and vice versa. Since BHT platelets have normal amounts of GP IIb-IIIa and, as we show, normal fibrinogen binding, the

possibility of abnormal membrane fluidity and membrane protein-cytoskeletal protein interactions needs to be investigated.

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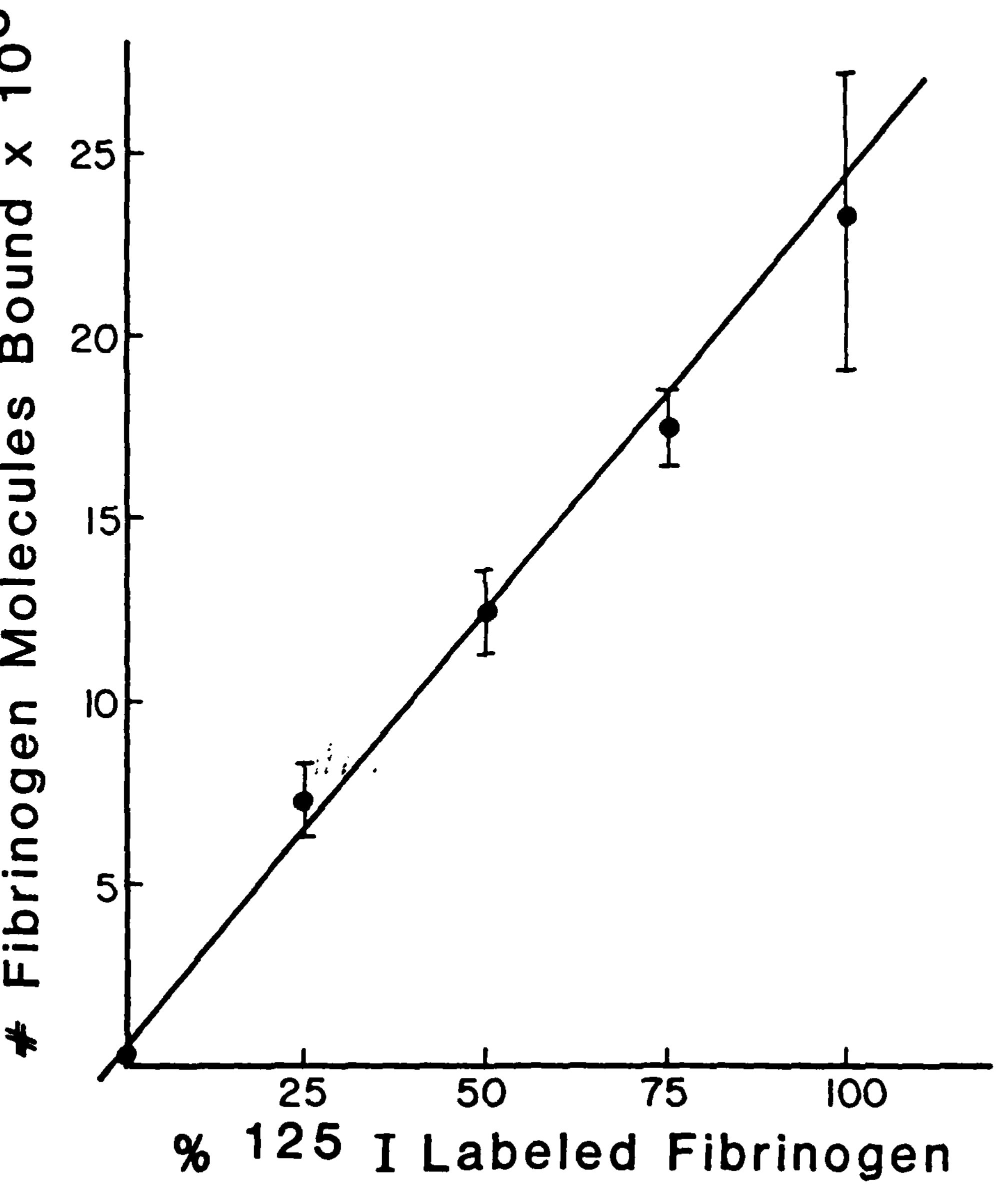
LEGENDS

Figure 1. Aggregation tracings of BHT and normal dog platelets in response to 1×10^{-5}M ADP. The only response noted with BHT platelets was a decrease in percent transmission indicative of shape change.

Figure 2. Competitive inhibition of ^{125}I- labeled fibrinogen binding. The total amount of fibrinogen was constant with only the percentage of ^{125}I- fibrinogen being varied. Unlabeled fibrinogen was able to compete with labeled fibrinogen for binding to receptors, after ADP stimulation, directly proportional to the amount of unlabeled fibrinogen present, which indicates specific binding to membrane receptors.



Fibrinogen Molecules Bound x 10^3
25
20
15
10
5
25
50
75
100
% 125 I Labeled Fibrinogen

FIG 2

COMPARATIVE FIBRINOGEN BINDING (MOLECULES/PLATELET)
IN BASSET HOUND HEREDITARY THROMBOPATHY

	Normal Dog Platelets		BHT Platelets	
Type Fibrinogen	22°C	37°C	22°C	37°C
BHT (dog)	20939 ±3269	7385 ±507	21748 ±3970	6461 ±2028
Normal (dog)	23217 ±299[illegible]	7893 ±704	25315 ±841	7283 ±288
Human	24942 ±5596	7384 ±2327	25408 ±4134	8197 ±2211
Mean Binding All types of Fibrinogen	23033 ±3971	7554 ±1267	23972 ±3612	7573 ±1848

Table 1

Number of molecules of ^{125}I- labeled fibrinogen bound to washed platelets from BHT and normal control dogs. Platelets were stimulated with ADP (1×10^{-5}M, final concentation) at 22°C and 37°C. Purified fibrinogen preparations of indicated types were used. Both homologous and heterologous results were averaged. Values are expressed as mean ± SD, (Three affected Basset Hound and three normal dog samples were run in triplicate).

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CHAPTER 5

SUMMARY

Normal hemostasis is accomplished through a complex set of reactions that involve platelets, the vascular wall, and the coagulation factors. Central to the hemostatic process is the role of the platelet which includes its ability to change shape, adhere to subendothelial tissue, release storage pool granule contents, and to aggregate into viable and stable platelet plugs. For some platelet stimuli, there must be a functional receptor on the platelet membrane to which the agonist binds and the stimulus is biochemically transmitted to the platelet interior. Microtubule mediated concentration and release of platelet granules follows, and finally, surface fibrinogen receptors are exposed and interact with fibrinogen thereby mediating aggregation. It has been shown that the platelet membrane fibrinogen receptor is a calcium-dependent heterodimer complex of glycoproteins IIb and IIIa (GP IIb-IIIa). Dysfunction of any of the above series of events can cause platelet aggregation abnormalities. A colony of Basset Hounds with a platelet aggregation defect was identified and various studies have been performed to attempt to pinpoint the exact defect. The results of these studies were reported in the preceding chapters.

As seen in chapter 1, platelets from dogs with Basset Hound Hereditary Thrombopathy are capable of releasing their storage pool granule contents in normal quantities, however, they do so much more rapidly than normal dog platelets. This seemingly unsynchronized release may have deleterious effects on the overall aggregation reaction and should be investigated further. In chapter 2, the platelet membrane and total platelet protein and glycoprotein content were shown to be normal, with an apparent defect in lipid content which will require more analytical techniques to fully establish. Finally, in chapter 3, it was shown that platelets from BHT affected dogs were able to bind fibrinogen in normal amounts when compared to normal dog platelets. To summarize, it was shown that BHT platelets release normal amounts of storage pool contents, they contain normal amounts of the GP IIb-IIIa complex, and they bind normal amounts of radiolabeled fibrinogen. However, they still fail to aggregate.

Possibilities that would explain the defect in light of the presented data would include: 1) the rapid release of storage pool contents is responsible for unsynchronizing the biochemical events necessary for a normal platelet aggregatory response. Perhaps if storage pool granule contents are released too early in the complex chain of events, they simply might not be in the right place at the right time; 2) the presence of an abnormal intracellular calcium content or flux after stimulation. Calcium

sequestration, movement, and resequestration are necessary events for proper muscle cell function and are also essential to normal platelet function. Abnormal sequestration or movement of calcium within BHT platelets would adversely affect several biochemical mechanisms which include microtubule reorganization, myosin phosphorylation, and stimulation of protein kinase C and phospholipase C. Since some of these calcium dependent events are directly related to secretion and aggregation, investigating this alternative could provide data which would better explain platelet responses after stimulation; and 3) abnormal membrane fluidity or lipid-protein interactions caused by abnormal lipid content which would adversely affect receptor mobility. Receptor mobility appears to be part of the normal course of events after stimulation. Painter et al. (1985) proposed that a small population of GP IIb-IIIa does not bind fibrinogen, but rather is associated with the platelet cytoskeleton. After stimulation and fibrinogen binding, the GP IIb-IIIa that does bind fibrinogen moves to become closely associated with the nonfibrinogen binding GP IIb-IIIa population. The final association presumably provides a bridge between the cytoskeletons of adjacent platelets. Another alternative related to abnormal lipid content would be that when some lipids are present in cell membranes the membranes are more rigid, and their presence may affect cell membrane charge. If this were the case perhaps an abnormally charged or rigid membrane would

disallow a conformational change in the GP IIb-IIIa complex after stimulation and fibrinogen binding. However this may not be the case in view of normal clot retraction in BHT. Yet another possible explanation for the platelet defect, which was previously discussed, may be an abnormal membrane glycoprotein-cytoskeletal interaction which is mediated by sulfhydryl and/or disulfide groups. These groups are implicated at least circumstantially in normal platelet function.

Although the exact molecular defect in BHT remains to be elucidated, we do now know a little more about BHT and normal platelets and by using this animal model, we can perhaps better understand the mechanisms of platelet aggregation.

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